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ARTICLE

Antidiabetic efficacy of sea fish oil in ameliorating hyperglycaemia by enhancing FFAR1, GLP-1 and inhibiting DPP-4 signalling in the pancreatic tissues of high lipid diet and streptozotocin-induced type 2 diabetic rats

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ABSTRACT Eicosapentaenoic acid and docosahexaenoic acid are n-3 fatty acids that are highly available in sea-fishes. Many studies have revealed that n-3 fatty acids play antidiabetic activity. In this study antidiabetic activity of the three sea fishes oils, 'volavetki' (*Panna microdon,* Bleeker 1849), 'ruli' (*Coilia dussumieri*, Valenciennes 1848), and 'tapra' (*Opisthopterus tardoore,* Cuvier 1829), as well as three fresh-water fishes oils, 'bata' (*Labeo bata,* Hamilton 1822), 'folui' (*Opisthopterus tardoore,* Cuvier 1829), and 'mourala' (*Amblypharyngodon mola,* Hamilton 1822) evaluated high lipid diet (HLD) and STZ-induced type 2 diabetes mellitus (T2DM) rats. The GC-MS analysis revealed high n-3 fatty acid content in volavetki oil. Supplementation of sea-fish oils and antidiabetic effect was monitored by fasting plasma blood glucose and HbA1c. Also, insulin, c-peptide, glucagon-like peptide-1, dipeptidyl peptidase-4, free fatty acid receptor 1, glucokinase, glucose-6 phosphatase, superoxide dismutase, glutathione peroxidase, catalase, malondialdehyde, plasma total cholesterol, low density lipoprotein, triglycerides, high density lipoprotein, c-reactive protein, total protein, alkaline phosphatase, and histopathology of pancreatic tissues were evaluated. Out of six fish oils, oral supplementation with volavetki oil resulted in significant reduction of plasma blood glucose, HbA1c of T2DM rat. All other parameters in T2DM rats were returned to near normally by treatment of volavetki oil. These findings strongly indicate that volavetki oil possess antidiabetic activity.
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Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder marked by hyperglycaemia and abnormalities in glucose, protein, and lipid metabolism, resulting in insulin production, action, or both (Gardner et al. 2022; Uzzaman and Ghaffar 2017; Li et al. 2022). T2DM and its major sequelae, like nerve damage (Scheen 2021), renal disease, and cardiovascular disease (Zhou et al. 2018; De et al. 2018), have raised society's medical burden dramatically in recent decades (Endo and Cui 2018). According to the 10th edition of the International Diabetes Federation (IDF 2021), the global prevalence of diabetes is 463 million (9.3%) in 2019, to be risen 578 million (10.2%) by 2030 and 700 million (10.9%) by 2045 (Rylander et al. 2014; Oloyede et al. 2015; Yun and Ko 2021). Studies showed that the prevalence of T2DM has become a serious public health concern worldwide, particularly in underdeveloped nations (Rahimi et al. 2017). Insulin, dietary modifications, and oral hypoglycaemic agents (OHAs) are all used to treat diabetes mellitus (Moon et al. 2017; Chow et al. 1995). There is several OHAs that stimulate insulin from beta cells includes dipeptidyl peptidase-4 (DPP-4) inhibitors (gliptin), glucagon-like peptide-1 (GLP-1, exenatide) analogue (Lin et al. 2015; Ren et al. 2020), free fatty acid receptor 1 (FFAR1, TAK-875 analogue (Li et al.2018; Yabuki et al. 2013). Besides these OHAs other examples sulfonylureas, biguanides, and other glucose-lowering drugs are expensive and have been related to increased body weight and hypoglycaemia with other slew of adverse effects like nausea, skin rashes, liver disease, heart failure diarrhoea, and so on (Idm'hand et al. 2020; Khan et al. 2021).

Researchers are trying to develop alternative less costly management strategies against T2DM (Plows et al. 2019; Zhang et al. 2022). Pyne et al. (2021) proved that freshwater fish eaters are more likely to develop T2DM than sea fish eaters. However, it is uncertain if eating sea fish contains a protective impact on T2DM (Pyne et al. 2021). Studies identified some antidiabetic sea fishes like salmon (*Salmo salar*, Linnaeus, 1758), tuna (*Longtail tuna*, Bleeker, 1851), grouper fish (*Malabar grouper*, Bleeker, 1874), etc. (Nanri et al. 2011). American Diabetes Association (ADA) recommended those fishes for consumption twice in a week for antidiabetic supplementation (Buse et al. 2020; Davies et al. 2018). Because the number of natural antidiabetic drugs and sources of food supplementation from sea-fish or marine resources are limited, the present study was aimed to search for new possible antidiabetic agents based on DPP-4 inhibitor, GLP-1 agonist, and FFAR1 agonistic action of oils from fish species (Holt et al. 2021; Hernandez-Caceres et al. 2019; Caroleo et al. 2021).

Materials and methods

Chemicals

Major biochemical parameters were measured using ELISA kits like insulin, c-peptide (Monobind, Lake Forest, CA, USA), FFAR1, DPP-4, GLP-1, glucokinase, and glucose-6-phosphatase (Develop, 214031, A3-South, PRC). Biochemical parameters were measured using standard kits: glucose (Atlas Medical, Cambridge, CB4 0WX), glycated haemoglobin (HbA1c), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), c-reactive protein (CRP), alkaline phosphatase (ALP), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Agappe Hills, Kerala, India). TRIS buffer, ethanol, hexane, isopropanol, and streptozotocin (STZ) were purchased from Sisco Research Laboratories (Mumbai, India), and Merck (Mumbai India). Glibenclamide (Supra Chemicals, Mumbai, India) was purchased locally.

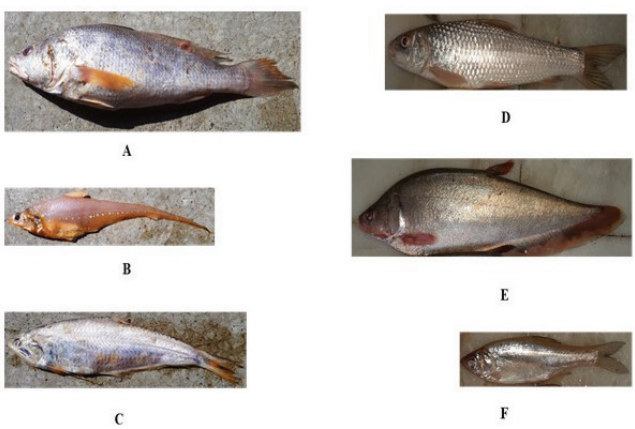

Figure 1. A 'volavetki' (*Panna microdon*, Bleeker 1849), B 'ruli' (*Coiliadussumieri*, Valenciennes 1848), C'tapra' (*Opisthopterus tardoore*, Cuvier 1829), D 'bata' (*Labeo bata*, Hamilton 1822), E 'folui' (*Opisthopterus tardoore*, Cuvier 18290), F 'mourala' (*Amblypharyngodon mola*, Hamilton 1822).

Sample collection

Three varieties of sea fishes 'volavetki' (*Panna microdon,* Bleeker 1849), 'ruli' (*Coilia dussumieri*, Valenciennes 1848), and 'tapra' (*Opisthopterus tardoore,* Cuvier 1829) were collected from direct fisheries person at the Digha mohona (a local sea fish market, 21° 37' 39.94" N, 87° 31' 10.74" E) the coastal area of Bay of Bengal (West Bengal, India) and three varieties of freshwater fishes 'bata' (*Labeo bata,* Hamilton 1822), 'folui' (*Opisthopterus tardoore,* Cuvier 1829), and 'mourala' (*Amblypharyngodon mola,* Hamilton 1822), (Kundu et al. 2015) were collected from local market (Kotwali bazar) at Midnapore town (22°25'1.54"N, 87°19'39.26"E), West Bengal, India (Fig. 1). The fishes were identified at the Department of Zoology, Raja Narendra Lal Khan Women's College (Autonomous), Midnapore, West Bengal and the specimens (RNLKWC/ZOO/FISH/2019) were deposited in the Department of Zoology. After collection of fishes, cleaned up and chopped to dry in sunlight for few days. After completely drying, grinded and made fine dust for oil extraction.

The oil content of the above said fishes were extracted in the Soxhlet apparatus using solvent for 6 h according to the method of American Oil Chemists Society (AOCS) (AOCS 1993). At first 1 kg of each fish dust was dissolved in 10 L hexane:isopropanol (3:2, v/v) in airtight glass jar for 24 h then kept in shaker incubator at 37 °C, thereby, filtered through Whatman No.1 filter paper and filtrate was stored in desiccator. When need the filtrate was evaporated by a rotary evaporator at 40 °C. The concentrated part was the content of total oil (Mandal et al. 2019).

Chemical characterization of fish oils

Determination of oil content

Oil content (%) = (oil weight/ fish dust weight) × 100 (Mandal et al. 2019).

Acid Value

Acid value = V× N×56.1/W
Where, N = 0.1 (N) KOH.
V = Volume (mL) of KOH.
W = Weight of oil (g) (Mandal et al. 2019).

Saponification value in mg of KOH/g of oil

Saponification Value = V× N× 56.1/W
Where, V = Volume (mL) of 0.5 (N) HCl.
N = KOH 0.5 (N).
W = Weight of oil (Mandal et al. 2019).

Fatty acid percentage by GC-FID

Fatty acid methyl esters (FAMEs) were prepared from 2 ml each oil of 3 sea fishes and 3 freshwater fishes separately as per our earlier work of Mandal et al. (2021) and followed by AOCS method (AOAC 1995). GC-FID was performed by GC-FID equipment (Agilent 7890, USA) with a SP 2560 (75 m x 0.18 mm x 0.14 mm) column. During analysis the condition of equipment was as follows: carrier gas (hydrogen) flow rate was 0.6 ml/min; injector temperature 250 °C; split ratio 1:100; oven program 140 °C to 220 °C; temperature of flame ionization detector 260 °C, zero air, and injection volume of 1 ml. The percentage of fatty acids were quantified by Supelco 37 FAME Mixture (Sigma 47,885-U) as a reference standard and obtained the percentage of fatty acids in oil separately (Mandal et al. 2021; Kh et al. 2022).

Care of animals

Fifty-five male albino rats (Wistar adult, pathogen free and healthy) having body weight (b.w.) of 220 ± 15 g and age average 45 days, were procured from the Centre for Translational Animal Research, Bose Institute, Kolkata. Rats were kept in normal laboratory conditions for 2 weeks prior to experimentation. The rats were housed with 3 rats/cage and another 2 rats/cage in a temperature-controlled room (22 ± 2 °C) with 12-12 h dark-light cycles at a humidity of 50 ± 10%. They were provided with standard food and water *ad libitum*. Rats were cared as per our standard operating procedure of the guiding principles for care and use of animals prepared by Institutional Animal Ethics Committee (IAEC) (meeting number-15/IAEC (05)/RNLKWC/2019) and the IAEC was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Animal Husbandry, Government of India.

Table 1. List of food ingredients of High Lipid Diet (HLD) diet.

Component	g
Corn starch	40
Casein	20
Bengal gram flour	15
Milk powder	-
Sucrose	10
Coconut Oil	13
Mineral mixture	0.5
Cholesterol	0.5
Vitamin premix	0.5
Salt	0.5
Total	*100*

Induction of T2DM in rats

All rats were provided HLD (Table 1) for 45 days to promote quick weight gain (Khatun et al. 2019). After 45 days, T2DM was achieved by a single intraperitoneal injection (i.p.) of STZ (40 mg/kg b.w. dissolved in 0.5 ml buffer (0.1 M sodium citrate, pH 4.5). HLD and water intake were closely monitored daily with measuring whole fasting blood glucose (WFBG) from tail vein of rat by glucometer. After 72 h of STZ injection, fasting blood glucose level reached above 126 mg/dl to consider T2DM (Khadke et al. 2020; Karigidi and Olaiya 2019).

Experiments on T2DM rats

The rats were divided into eleven groups of five rats each (n = 5). The duration of fish oil supplementation was 28 days and the day of STZ injection was considered first day of experiment. The rats were grouped as follows: control rats were fed HLD without STZ injection, vehicle control rats were fed HLD with single shot of 0.5 ml 0.1 M citrate buffer without STZ injection i.p., and other nine groups of rats were induced T2DM followed by point 2.7, thereby, volavetki oil (VOL), ruli oil (RUL), tapra oil (TAP), bata oil (BAT), folui oil (FOL), mourala oil (MOU), and olive oil (OLI) were orally fed at the dose of 600 mg/ kg body weight to diabetic + VOL group, diabetic + RUL group, diabetic+ TAP group, diabetic + BAT group, diabetic + FOL group, diabetic + MOU group, and diabetic + OLI group, respectively, and eleventh group fed glibenclamide at the dose of 0.5 mg/kg b.w. (Khatun et al. 2019; Khadke et al. 2020).

Estimation of blood glucose and HbA1c level

Blood samples from rat's tail vein collected on days 0, 3, 14, and 28 in an overnight fasting state. Blood glucose was measured by a glucometer and HbA1c was estimated by standard kit method using semiauto analyser (Karigidi et al. 2019).

Scarification blood and tissues collection

At the end of the experimental period, rats were sacrificed after overnight fasting. Rats were anesthetized with ketamine 50 mg/kg b.w. i.p. and blood was collected from the hepatic vein. Plasma was prepared from blood by centrifugation and pancreatic tissues were collected and preserved at -20 °C (Roy et al. 2014).

Estimation of lipid profile and toxicity markers

TC, TG, LDL, HDL, GOT, GPT, CRP, and ALP were

Table 2. Characterization of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil.

Parameters	VOL	RUL	TAP	BAT	FOL	MOU
Oil percentage (g %)	11.55 ± 0.61	23.14 ± 1.14 *	17.48 ± 1.21 *	22.57 ± 2.07 *	21.93 ± 1.78 *	32.97 ± 2.27*
Acid no (mg KOH/g)	0.7 ± 0.11	1.23 ± 0.18 *	5.13 ± 0.41 *	13.83 ± 0.18 *	14.63 ± 0.24 *	17.69 ± 0.90*
Saponification value (mg KOH/g)	113.13 ± 1 .07	148.15 ± 2.23 *	196.87 ± 2.94 *	321.57 ± 1.72 *	260.9 ± 2.65	357.93 ± 1.33 *

Data were expressed as mean ± SE (n = 3) and analysed by multiple comparison two tail "t" test. * p< 0.05 when compared with VOL.

measured using standard kit by semi-autoanalyzer as described earlier (Khatun et al. 2019; Khatun et al. 2021; Srujana et al. 2018).

Estimation of antioxidant enzyme activities of pancreatic tissue

Superoxide dismutase (SOD) activity

The pancreas was homogenized in ice-cold 100 mM Tris-cacodylate buffer to yield concentration of 50 mg/ml and centrifuged at 10 000 rpm for 20 min at 4 °C. One unit of SOD was defined as the enzyme activity that inhibited the autooxidation of pyrogallol by 50%. The SOD activity was expressed as U/mg of tissue/min (Giri et al. 2019; Das et al. 2016).

Catalase (CAT) activity

Pancreatic tissues were homogenized in 0.05 M Tris-HCl buffer (pH 7.0) at a tissue concentration of 50 mg/ml. These homogenates were centrifuged separately at 10 000 rpm at 4 °C for 10 min. CAT was measured in tissue supernatant by our laboratory established method and reading of absorbance was noted using a spectrophotometer at 240 nm (Giri et al. 2019; Das et al. 2016).

Quantification of malondialdehyde (MDA) level

The level of MDA was determined by the method of Mandal and co-workers (2015). Measurements were carried out by a spectrophotometer at 535 nm and values are expressed nmol/mg of pancreatic tissue.

Reduced glutathione (GSH) level

Quantification of GSH was performed by pancreatic tissue homogenate (0.2 M sodium phosphate buffer, pH 8.0) mixed with dithio-bis-nitrobenzoic acid according to the standard method using spectrophotometer at 405 nm. The levels of GSH were expressed as μg of GSH/mg pancreatic tissue (Mandal et al. 2015).

Estimation of major biochemical parameters

Insulin, c-peptide (Shapiro et al. 2021), and GLP-1 (Ren et al. 2020) levels in plasma, as well as FFAR1 (Kaku et al. 2015), and DPP-4 (Dono et al. 2022) were measured by standard kit methods by an ELISA plate reader.

Glucokinase of pancreatic tissue and glucose-6-phosphatase of liver tissue were measured standard kit method by an ELISA plate reader (Cui et al. 2018).

Table 3. Fatty acids composition (mg/100 g) of the fish oils used in the study.

Fatty acids	VOL	RUL	TAP	BAT	FOL	MOU
Lauric acid C12:0	10.52±0.01	18.02±0.01*	15.34667±0.01*	15.11±0.005*	6.34±0.005*	9.77±0.01*
Myristic acid, C14:0	77.63±0.01	291.47±0.02*	187.67±0.005*	192.18±0.01*	87.21±0.005*	198.19±0.005*
Palmitic acid, C16:0	599.55±0.005	979±0.58*	800.01±0.005*	904.49±0.01*	646.42±0.01*	609.31±0.005*
Stearic acid, C18:0	296.93±0.005	395.02±0.01*	396.49±0.005*	166.89±0.005*	223.79±0.005*	204.9±0.005*
Myristoleic acid, C14:1	1.72±0.008	3.22±0.01*	2.35±0.005*	12.07±0.005*	9.51±0.014*	14.78±0.01*
Oleic acid, C18:1n9c	240.48±0.01	269.0067±0.008*	406.84±0.01*	324.22±0.01*	431.94±0.005*	426.87±0.005*
Linolenic acid, C18:3n3	3.29±0.01	18.75±0.005*	15.55±0.005*	305.58±0.005*	72.29±0.005*	211.52±0.005*
Arachidonic acid, C20:4n6	105.15±0.01	70.14±0.01*	69.22±0.005*	63.42±0.02*	102.73±0.02*	56.19±0.005*
Eicosapentaenoic acid, C20:5n3	132.61±0.005 *	221.34±0.01*	156.46±0.02*	63±0.58*	30.75±0.005*	46.3±0.06*
Docosapentaenoic acid, C22:5n-3	46.34±0.005	59.3±0.005*	53.27333±0.01*	39.14±0.02*	25.64±0.02*	16.75±0.005*
Docosapentaenoic acid, C22:5n-6	321.9±0.005	255±0.58*	248.8±0.005*	318.3±0.005*	172.8±0.01*	291.2033±0.02*
Docosahexaenoic acid, C22:6n3	328.49±0.005	265.15±0.02*	227.06±0.01*	76.14±0.01*	60.28±0.02*	56.37±0.02*

Data were expressed as mean ± SE (n = 3) and analysed by multiple comparison two tail "t" test. * = p < 0.05 when compared with VOL.

Table 4. Effect of fish oils, olive oil, and glibenclamide on body weight and Pancreatic Somatic Index of HLD- and STZ-induced T2DM rats.

Parameters	Control	Vehicle control	Diabetic	Diabetic + VOL	Diabetic + RUL	Diabetic + TAP	Diabetic + BAT	Diabetic + FOL	Diabetic + MOU	Diabetic + OLI	Gliben-clamide
IBW (g)	263 ± 7.81	268.40 ± 4.84*	262.8 ± 3.29	261.4 ± 6.10	262.20 ± 4.78	264.19 ± 4.22*	256.8 ± 4.62	260.61 ± 3.97	262.8 ± 4.98	268.82 ± 5.69*	260.8 ± 2.99
FBW (g)	283 ± 9.29*	293.2 ± 5.39*	250 ± 6.09	275.32 ± 6.78*	272.7 ± 4.04*	273 ± 3.10*	262.2 ± 4.46	264.9 ± 3.61	259 ± 4.95	271.2 ± 6.30	272 ± 4.40*
Difference	20 ± 1.84*	24.80 ± 2.08*	2.8 ± 0.40	13.92 ± 3.26*	10.50 ± 2.89*	8.81 ± 2.06*	5.4 ± 0.81*	4.29 ± 0.83	1.6 ± 0.87	2.38 ± 0.69	11.2 ± 1.91*
Increase or decrease (%)	7.57	9.25	1.12	5.35	4.05	3.37	2.11	1.66	0.62	0.87	4.28
Weight of the pancreas	1.83 ± 0.05*	1.90 ± 0.19*	1.28 ± 0.15	1.81 ± 0.14*	1.54 ± 0.05	1.68 ± 0.11	1.32 ± 0.12	1.24 ± 0.09	1.36 ± 0.16	1.19 ± 0.15	1.44 ± 0.13
PSI	0.65	0.65	0.61	0.72	0.63	0.69	0.55	0.51	0.55	0.48	0.59

IBW: Initial body weight; FBW: Final body weight; PSI: Pancreatic Somatic Index. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. * $p < 0.05$ when compared with diabetic rats.

Histological study of pancreatic tissue

A small rectangular piece of pancreatic tissue was immersed in 10% buffered formalin solution to preserve the tissue structure. The tissue was then dehydrated and embedded in paraffin wax to facilitate sectioning. Tissue section was cut of 5 μm thickness using a rotary microtome and stained with haematoxylin-eosin counter stain. The stained tissue sections were observed under a binocular inverted phase contrast microscope to study the histological structure (Khatun et al., 2019; Oliyaei et al. 2021).

Data analysis

Data were expressed as mean ± standard error (n = 5) and analysed by multiple comparisons two-tail "t" test by using statistical package Origin 6.1 (OriginLab).

Results

Oil content

Volavetki sea fish had the lowest oil content (11.55%, w/w). The oil content (w/w) in other fishes were 17.48%, 21.93%, 23.14%, 22.57%, and 32.97%, in fishes tapra, folui, bata, ruli, and mourala, respectively (Table 2).

Acid value

The acid value is a measure of the free fatty acids in the oil. Acceptable levels of acid value in oil should be below 0.6 mg KOH/g (measured in potassium hydroxide per gram). FOL, BAT, and MOU had significantly ($p < 0.05$) higher acid values than sea fish oils (Table 2). Among all oil samples, MOU showed the highest (17.69 ± 0.9 mg KOH/g) acid value and VOL showed the lowest (0.7 ± 0.11 mg KOH/g) acid value.

Saponification value

A higher saponification value indicates a high proportion

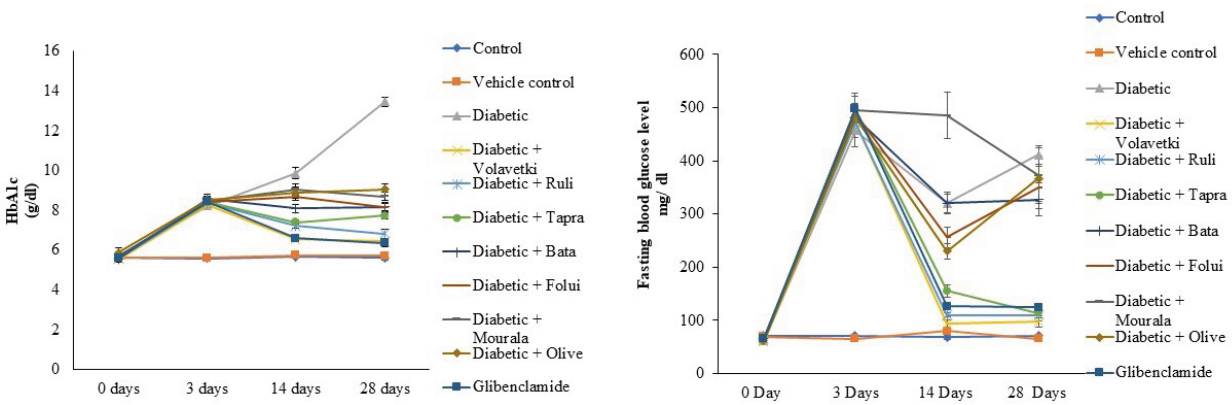

Figure 2. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on HbA1c and fasting blood glucose level (mg/ dl) of HLD- and STZ-induced T2DM in male albino rats. Data were expressed as mean ± SE (n = 5).

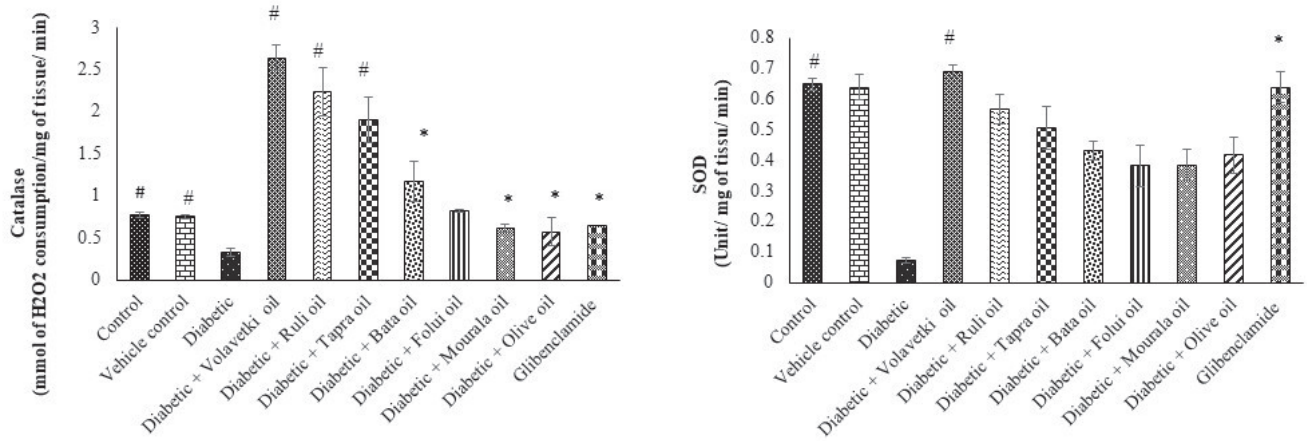

Figure 3. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide of HLD- and STZ-induced T2DM male albino rats, intervention of catalase (mmol of H2O2 consumption/mg of pancreatic tissue/min) activity and SOD (mmol of H_2O_2 consumption/dL of pancreatic tissue/min) activity. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # $p < 0.01$, and * $p < 0.05$ when compared with diabetic rats.

of lower fatty acids since saponification value is inversely proportional to the chain length of the fatty acids. Therefore, the shorter the average chain length (C4-C12) the higher the saponification number. VOL had a significantly ($p < 0.05$) lower saponification value (113.13 ± 1.07 mg KOH/g) than other investigated fish oils (Table 2).

Fatty acids composition

Twelve fatty acids were classified and measured from six fish oil samples (Table 3). Dominant fatty acids of all the studied sea fishes oils were C16:0 (palmitic acid), C18:0 (stearic acid), C20:5, n-3 (eicosapentaenoic acid, EPA), C22:5, n-6 (Osbond acid), C22:6, n-3 (docosahexaenoic acid, DHA) and in freshwater fish oils were palmitic acid, C18:1, n-9 (oleic acid), and C18:3, n-3 (linolenic acid, LA). VOL contained a very different fatty acids profile compared to all other fish oils: it contained low level of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and the highest level of polyunsaturated fatty acids (PUFA) when compared with the other fish oils.

Assessment of average body weight and pancreatic weight

VOL treated rats showed an increase ($p < 0.05$) in feed consumption as compared to diabetic rats. The other fish oil-treated groups of rats, olive oil-treated and glib-

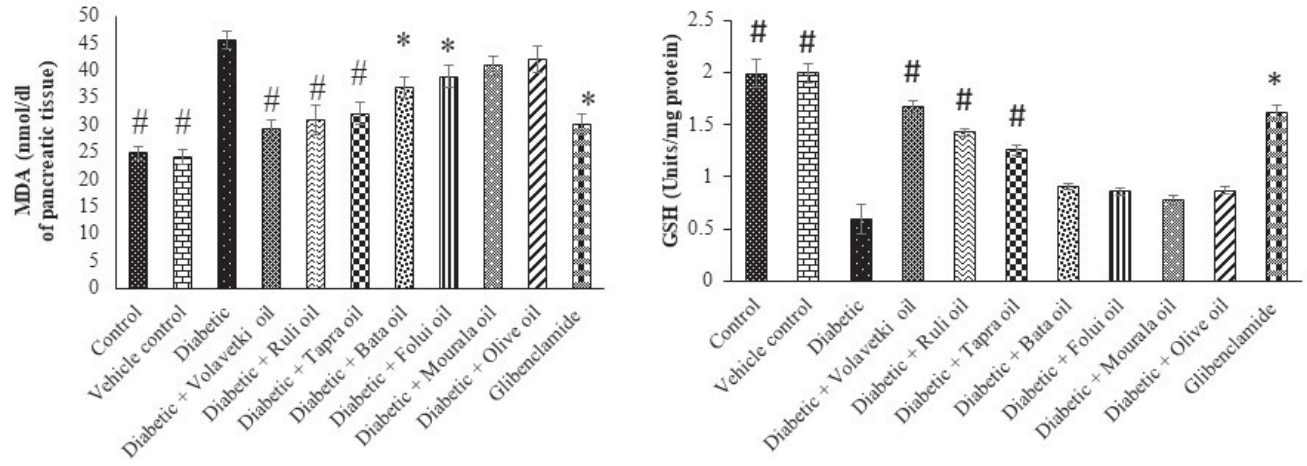

Figure 4. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on of HLD- and STZ-induced T2DM male albino rats intervention of MDA (nmol/dl) and GSH (U/mg protein) activity. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # $p < 0.01$ and * $p < 0.05$ when compared with diabetic rats.

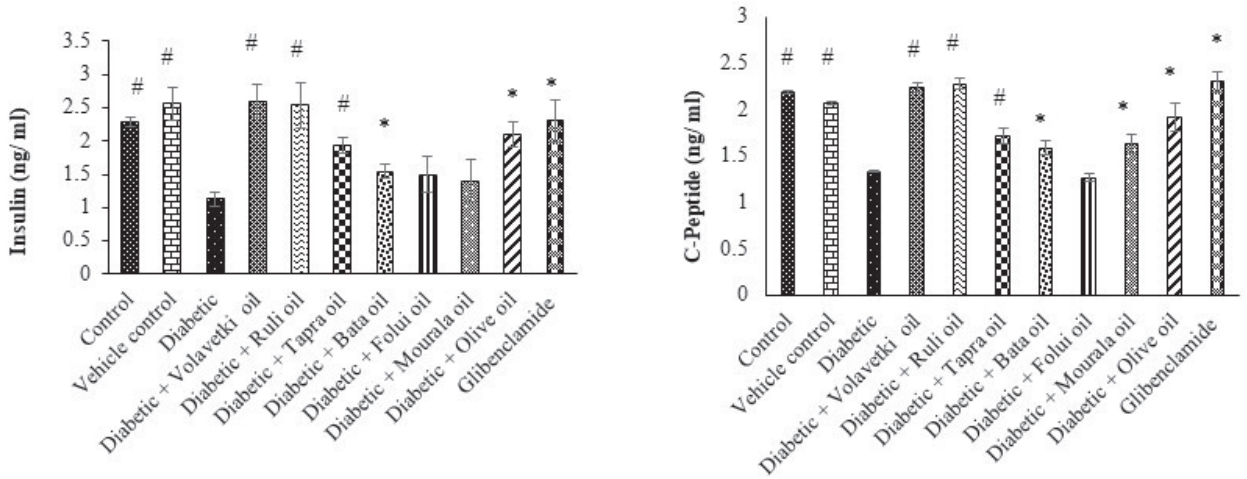

Figure 5. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on of HLD- and STZ-induced T2DM male albino rats of intervention of plasma insulin and c-peptide level. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # $p < 0.01$ and * $p < 0.05$ when compared with diabetic rats.

enclamide-treated rat groups showed significantly ($p< 0.05$) decreased feed intake and water intake as compared to the diabetic group. Whereas body weight was significantly ($p< 0.05$) elevated in VOL-treated groups as compared to the diabetic group. The organ weight of experimental animals is shown in Table 4. Diabetic rats showed decreased pancreatic tissue weight, as compared to the control, vehicle, VOL-, and RUL-supplemented rats. Whereas weight of pancreatic tissues significantly ($p< 0.05$) decreased in the BAT, FOL, MOU, OLI, and

Table 5. Effect of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on the estimation of biochemical parameters of HLD- and STZ-induced T2DM rats.

Parameters	Control	Vehicle control	Diabetic	Diabetic + VOL	Diabetic + RUL	Diabetic + TAP	Diabetic + BAT	Diabetic + FOL	Diabetic + MOU	Diabetic + OLI	Glibenclamide
Glucose (mg/dl)	71.4 ± 1.78#	65.2 ± 5.11#	411.2 ± 17.59	97.2 ± 10.37#	109.6 ± 9.97#	113.4 ± 8.54#	327.4 ± 30.83*	349.2 ± 39.46	372 ± 51.56	366.4 ± 42.92	124.6 ± 7.57*
HbA1c (g/dl)	5.62 ± 0.11#	5.72 ± 0.10#	13.44 ± 0.23	6.44 ± 0.11#	6.82 ± 0.22#	7.74 ± 0.20#	8.16 ± 0.20*	8.14 ± 0.26*	8.68 ± 0.21*	9.04 ± 0.30*	6.36 ± 0.20*
Plasma GPT (U/ml)	27.24 ± 1.05#	25.26 ± 1.42#	78.9 ± 3.17	31.85 ± 1.60#	36.71 ± 1.08#	49.87 ± 2.27#	54.80 ± 3.18*	54.68 ± 3.66*	61.54 ± 3.63*	79.78 ± 4.45	29.5 ± 1.48*
Plasma GOT (U/mL)	16.08 ± 1.43#	18.62 ± 1.68#	82.22 ± 4.18	27.88 ± 1.50#	30.34 ± 1.54#	34.34 ± 2.22#	65.96 ± 2.90*	68.06 ± 2.74*	72.64 ± 3.59	78.21 ± 2.80	27.10 ± 1*
Total protein (g/dl)	7 ± 1.55	6.74 ± 2.09	13 ± 2.02	6.08 ± 0.98	8.56 ± 0.92	10.22 ± 1.95	11.36 ± 1.92	11.03 ± 1.12	12.22 ± 1.81	13.6 ± 1.62	7.1 ± 1.26*
ALP (IU/L)	111.86 ± 1.78#	114.36 ± 3.16 #	307.44 ± 3.39	144 ± 2.63#	157.74 ± 2.04#	165.44 ± 3.32#	208.4 ± 4.41*	216.14 ± 3.32*	272.84 ± 4.82*	283.3 ± 3.27*	139.04 ± 5.73*
CRP (mg/L)	3.26 ± 0.51#	3.088 ± 0.56#	9.486 ± 1.16	3.82 ± 0.76#	4.22 ± 1.09	6.682 ± 1.29	8.14 ± 1.15	9.82 ± 2.43	11.36 ± 1.93	11.52 ± 1.83	3.28 ± 0.36 *
TC (mg/dl)	116.01 ± 2.75#	116.70 ± 4.94#	179.51 ± 5.92	134.79 ± 1.28#	141.27 ± 3.92#	157.8 ± 4.91	161.11 ± 8.84	168.04 ± 7.12	172.94 ± 9.37	173.49 ± 5.73	130.95 ± 2.50 *
TG (mg/dl)	100.56 ± 4.76#	100.09 ± 2.97#	134.12 ± 3.21	101.64 ± 4.22#	108.36 ± 3.39#	111.90 ± 5.57#	116.64 ± 4.16*	117.38 ± 3.99*	118.32 ± 3.71*	129.23 ± 2.34	102.21 ± 4.42 *
HDL (mg/dl)	44.89 ± 1.21 #	45.57 ± 1#	32.65 ± 1.67	43.92 ± 1.50#	38.90 ± 2.06	36.94 ± 2.95	36.82 ± 3.24	34.14 ± 1.82	33.25 ± 1.91	31.48 ± 1.83	40.75 ± 1.46 *
LDL (mg/dl)	65.3 ± 1.47#	65.25 ± 1.29#	121.33 ± 9.47	68.66 ± 2.01#	70.82 ± 1.28#	73.12 ± 2.30#	87.03 ± 3.36	91.96 ± 2.85*	92.22 ± 3.75*	94.19 ± 6.31*	64.76 ± 0.15 *

n: number of samples; Plasma GOT: Plasma glutamic oxaloacetic transaminase; Plasma GPT: Plasma glutamic pyruvic transaminase; CRP: C-Reactive Protein; ALP: Alkaline Phosphatase; TC: Total cholesterol; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # $p < 0.01$, and * $p < 0.05$ when compared with diabetic rats.

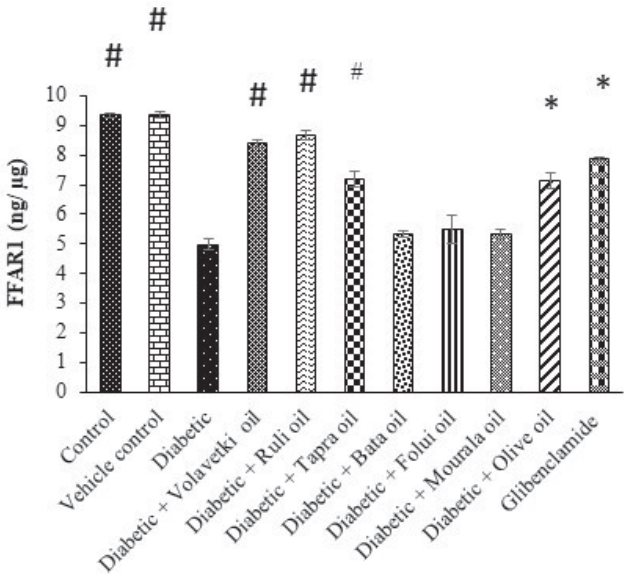

Figure 6. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on of HLD- and STZ-induced T2DM male albino rats, intervention of FFAR1 activity of pancreatic tissue. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # p < 0.01 and * p < 0.05 when compared with diabetic rats.

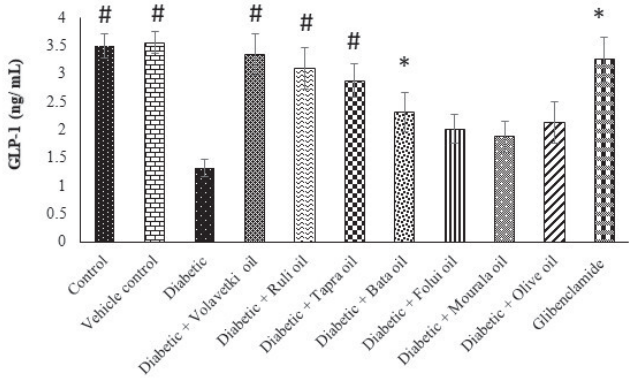

Figure 7. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on of HLD- and STZ-induced T2DM male albino rats, intervention of plasma GLP-1 activity. Data were expressed as Mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # p< 0.01 and * p< 0.05 when compared with diabetic rats.

glibenclamide groups as compared to the control, vehicle control, VOL, RUL, and TAP groups.

The VOL supplementation group intervention of showed significantly ($p < 0.05$) increased final body weight as compared to the diabetic group. Similarly, final body weight also significantly ($p < 0.05$) increased in control, vehicle control, VOL, RUL, TAP intervention groups as compared to diabetic, BAT, FOL, MOU, OLI, and glibenclamide groups. Whereas final body weight and pancreases weight decreased in diabetic, BAT, FOL, MOU, OLI, and glibenclamide treatment groups as compared to diabetic + VOL groups (Table 4).

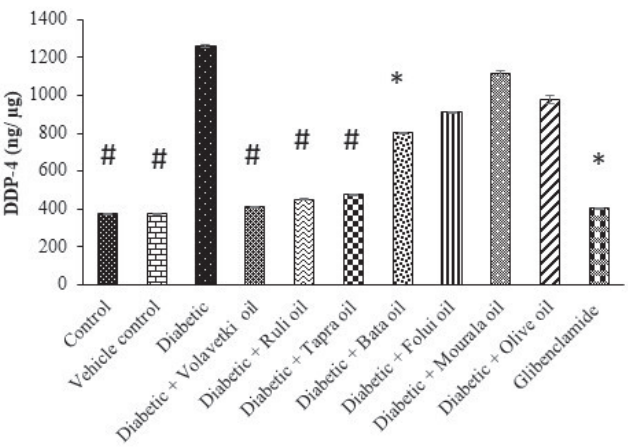

Figure 8. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on of HLD and STZ induced T2DM male albino rats, intervention of pancreatic DDP-4 activity. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # p < 0.01, and * p < 0.05 when compared with diabetic rats.

Estimation of blood glucose level, HbA1c, lipid profile and toxicity markers of plasma

Streptozotocin considerably increased blood HbA1c levels when compared to the control (Fig. 2). VOL-, RUL-, and glibenclamide-treated group had significantly decreased blood HbA1c as compared to the corresponding diabetic value. Concurrent administration of VOL, RUL, and glibenclamide nearly normalized blood HbA1c.

The effect of sea fish oils on blood glucose level are shown in Table 5. and Fig. 4. There was a decrease in the level of blood glucose of VOL- and RUL-treated groups when compared with the diabetic untreated group but significantly elevated when compared with the control group.

VOL-, RUL-, and TAP-treated rats had significantly lower plasma glucose as compared to diabetic rats. Mean value of plasma GPT and GOT of freshwater fish oils-treated groups were significantly higher than those treated with a sea fish oil. The mean ALP, total protein, and CRP level were higher in groups treated with a freshwater fish oil than in groups with a sea fish oil supplement.

The plasma lipid profiles of all experimental groups are shown in Table 5. Diabetic rats showed increased plasma TC, TG, and LDL level as compared to the control rats.

VOL-, RUL-, and TAP-treated group showed decreased plasma TC level as compared with diabetic group. Plasma

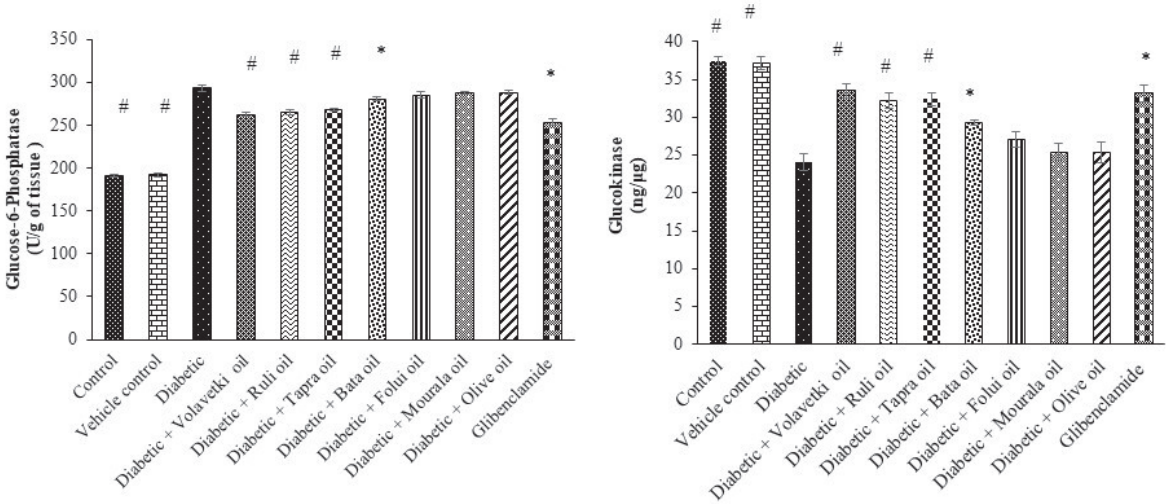

Figure 9. Effects of volavetki oil, ruli oil, tapra oil, bata oil, folui oil, and mourala oil, olive oil, and glibenclamide on of HLD- and STZ-induced T2DM male albino rats, intervention of glucose-6-phosphatase and glucokinase activity. Data were expressed as mean ± SE (n = 5) and analysed by multiple comparison two tail "t" test. # $p < 0.01$, and * $p < 0.05$ when compared with diabetic rats.

TG and LDL levels were also significantly decreased in VOL-treated as compared to the diabetic rats. All sea fish oil supplementation significantly elevated the plasma HDL level as compared to the diabetic rats.

Freshwater fish oil and olive oil-treated rats showed an increase in TC, TG, LDL and HDL levels compared to glibenclamide-treated rats. The plasma TC level was found to be comparable among glibenclamide- and sea fish oil-treated groups. Plasma TG, LDL, and HDL levels were higher in sea fish oil intervention groups compared to glibenclamide-treated rats.

Estimation of antioxidant enzymes activity of pancreatic tissue

The effect of VOL supplementation on hyperglycaemia-mediated oxidative stress in pancreatic tissues of streptozotocin-induced diabetic rats is shown in Figure 3 and 4.

Activities of antioxidant enzymes (CAT, SOD, and GSH) were significantly lower in diabetic rats when compared with the control and VOL-supplemented rats. VOL supplementation was able to lower the oxidative stress marker (MDA) level and raised the decreased CAT, SOD and GSH level in the pancreatic tissues of the diabetic rats.

Estimation of insulin, c-peptide, GLP-1 level in plasma and DDP-4, FFAR1, activity in pancreatic tissue

There was a significant increase in plasma insulin and c-peptide levels in VOL-treated rats as compared to the diabetic, olive oil-, and freshwater fish oil-treated groups (Fig. 5). VOL-treated rats have increased FFAR1 level in pancreatic tissue compared to the diabetic group (Fig. 6).

Diabetic rats showed increased pancreatic DPP-4 level as compared to the control rats. This pancreatic DPP-4 level decreased in VOL-treated rats as compared to the diabetic rats.

Plasma GLP-1 level was decreased in diabetic rats as compared to control rats. VOL and RUL supplementation significantly increased plasma GLP-1 level as compared with diabetic group (Fig. 7 and 8).

Estimation of glucokinase of pancreatic tissue, and glucose-6-phosphatase of liver tissue

Glucose-6-phosphatase is the rate-limiting enzymes controlling gluconeogenesis and glycogenolysis in the liver. These enzyme activities increased in diabetic rats compared to control group. Glucose-6-phosphatase levels of VOL supplemented rats decreased significantly compared to diabetic group. Similarly, glucokinase activities were also decreased in diabetic groups (Fig. 9). However, VOL supplementation significantly elevated glucokinase level compare with diabetic rats.

Histological study of pancreatic tissue

Histological examination of the pancreas of diabetic rats showed maximum destruction of the pancreatic islet and the acinar cells were swollen (Fig. 10C). In the pancreas of control and vehicle control rats, islets of Langerhans appeared organized (Fig. 10A-B). Microscopy examination of the pancreatic tissue of the VOL (Fig. 10D), olive oil (Fig. 10J), and glibenclamide groups (Fig. 10K) revealed that islets of Langerhans have a normal looking as in the control group. Partial destruction of the pancreatic islet was observed in the rest of the groups (Fig. 10). Histopathological examination of pancreatic tissue revealed

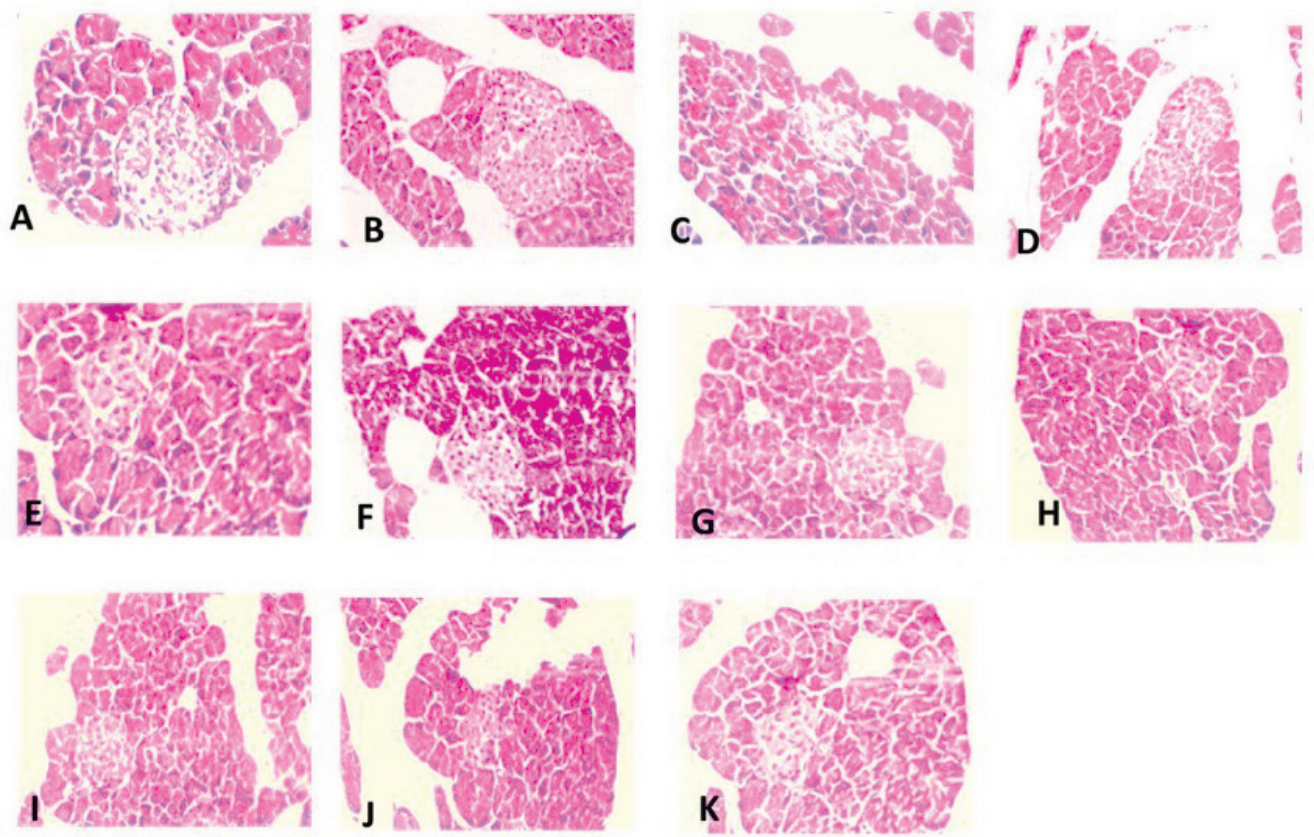

Figure 10. Microphotographs of histological changes in pancreas at 200x magnification of different treatment groups. A: Control; B: Vehicle control; C: Diabetic; D: Diabetic + VOL; E: Diabetic + RUL; F: Diabetic + TAP; G: Diabetic + BAT; H: Diabetic + FOL; I: Diabetic + MOU; J: Diabetic + OLI; K: Glibenclamide.

that VOL has the potential to prevent the degeneration of islets of Langerhans in pancreatic tissue (Fig. 10).

Discussion

Effects of various fish oils on hyperglycaemia in HLD- and STZ-induced T2DM rats have been investigated. Results enlightened that supplementation with sea fish oil enhanced insulin secretion, GLP-1 concentration, FFAR1 expression, DPP-4 inhibition, and diminution of glucose-6-phosphatase activity. Antidiabetic effect is fully involved in the GLP-1 secretion from intestinal cell and DPP-4 inhibition in pancreatic tissue.

Pathways that are targeted by sea fish oil supplementation for both synergistic and combinatorically tried to be identified. This can lead to discovery of new therapeutic benefits with new mechanistic target holding FFAR1 and agonist. Two mechanisms have been suggested: first mechanism is FFAR1 receptor activation, the second mode of action includes GLP-1, DPP-4, and glucose-6-phosphatase (Fig. 11).

Nowadays, for the treatment of T2DM many synthetic drugs as Metformin, Saxagliptin, Sitagliptin, Linagliptin, and Vildagliptin are used. However, these drugs are associated with side effects including kidney, urinary tract, and gastrointestinal infection, and many others (Biessels et al. 2021; Kadowaki et al. 2018; Oita et al. 2018; Rosenstock et al. 2019). With understanding the current scenario for the treatment of T2DM, outcomes from our study with a challenge of "one supplement, multiple targets' shifted from 'one supplement or drug, one target model". It gives a complementary approach in development of antidiabetic supplements.

In our previous study, we identified and evaluated the differences in blood glucose levels among individuals who consume sea fish and those who consume freshwater fish. Specifically, individuals who consume sea fish oil have a lower risk of T2DM compared to non-sea fish eaters. On the other hand, individuals who consume freshwater fish have a higher risk of T2DM (Pyne et al., 2021). In the current study, we extended our research by selecting three types of sea fish and three types of freshwater fish. We extracted oil from these fish and assessed their potential

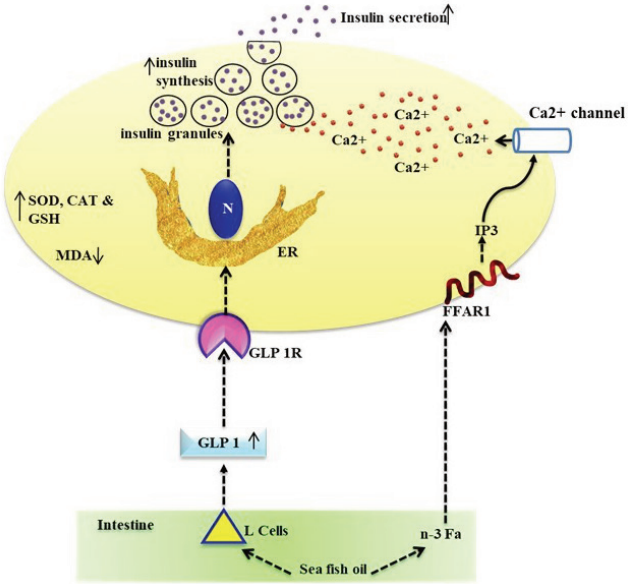

Figure 11. New mechanistic way of effects of sea fish oil on the increase of insulin and FFAR1 secretion.

antidiabetic activity. Additionally, we conducted a mechanistic investigation into the synergistic effects involving GLP-1, DPP-4, glucose-6-phosphate, and FFAR1 using an animal model of T2DM induced by a high-fat diet (HFD) and streptozotocin (STZ).

The supplementation of T2DM rats with 600 mg/kg body weight sea fish oil rich in DHA and EPA significantly reduced fasting plasma glucose levels and enhanced insulin secretion to highlighting its antihyperglycemic effects. Supplementation of VOL reveled better hyperglycaemic effects than other sea fishes (Bhaswant et al. 2015; Wang and Chan 2015; Winzell et al. 2006).

We have explored various mechanisms underlying the antihyperglycemic effects of sea fish oil. These include DPP-4 inhibition and the augmentation of plasma GLP-1 levels. DPP-4 inhibition plays a crucial role in reducing fasting blood glucose and enhancing insulin secretion. GLP-1, a protein that suppresses glucagon release and promotes insulin secretion, undergoes degradation due to heightened DPP-4 activity. This enzyme is prominently expressed in pancreatic beta cells, particularly during periods of oxidative stress. DPP-4 acts as a serine protease, cleaving the peptide bond between alanine and proline in GLP-1 (Parthan et al. 2018; Shankar et al. 2015; Ren et al. 2020; Rosenstock et al. 2019).

The study reveals that sea fish oil, particularly VOL, significantly reduces DPP-4 concentration in pancreatic tissue compared to other fish oil groups. Oxidative stress was evaluated by analysing lipid peroxidation and antioxidant enzyme activity in pancreatic tissue. The high-fat, low-dose STZ diet is established for inducing T2DM in rats through free radical generation (Fuchigami et al. 2020; Maeda et al. 2007; Ojuade et al. 2021).

Supplementing with VOL enhances antioxidant enzyme activity (SOD, catalase) in pancreatic tissue while decreasing lipid peroxidation. Sea fish oil raises diminished pancreatic GSH levels in the diabetic group, indicating reduced oxidative stress (Ahmed et al. 2014; Taheri et al. 2017; Kumar et al. 2014).

We explored FFAR1 activity in diabetic rats supplemented with VOL. VOL exhibits clear FFAR1 agonism, activated by long-chain unsaturated fatty acids like DHA and EPA. FFAR1 activation increases intracellular Ca^{2+} levels through inositol trisphosphate-induced protein kinase-C phosphorylation, stimulating insulin granule release (Wang et al. 2013; Borah and Das 2017; Benrahou et al. 2022).

In HLD and STZ-induced T2DM rats, lipid disorder abnormalities were corrected by sea fish oil. T2DM rats exhibited dyslipidaemia with elevated TC, TG, LDL, and reduced HDL, risking atherosclerosis and coronary disease (Akash et al. 2014; Hou et al. 2021; Jiang et al. 2015). VOL reduced TC, TG, LDL, while increasing HDL in T2DM rats, likely due to DHA and EPA's antidyslipidaemic effects.

Elevated liver toxicity enzymes (GOT, GPT, ALP) signal HLD and STZ-induced hepatocellular damage; VOL normalized these enzyme activities.

Our investigation into sea fish's antidiabetic potential in a high lipid diet and STZ-induced T2DM rat model uncovered substantial n-3 fatty acids (EPA, DHA) in VOL. It exhibits antidiabetic activity via DPP-4 inhibition, GLP-1 and FFAR1 agonism. This introduces a novel mechanism for identifying antidiabetic sea fish oil, warranting in-silico bioinformatics study for future mechanistic validation.

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